FEBS Letters 579 (2005) 5974–5981 FEBS 29976

Minireview

Approaches for chemically synthesized siRNA and vector-mediated RNAi

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Received 18 July 2005; revised 26 August 2005; accepted 28 August 2005

Available online 20 September 2005

Edited by Shou-Wei Ding

Abstract Successful applications of RNAi in mammalian cells depend upon effective knockdown of targeted transcripts and efficient intracellular delivery of either preformed si/shRNAs or vector expressed si/shRNAs. We have previously demonstrated that 27 base pair double stranded RNAs which are substrates for Dicer can be up to 100 times more potent than 21mer siRNAs. In this mini-review we elaborate upon the rationale and design strategies for creating Dicer substrate RNAs that provide enhanced knockdown of targeted RNAs and minimize the utilization of the sense strand as RNAi effectors. Expression of shRNAs or siRNAs in mammalian cells can be achieved via transcription from either Pol II or Pol III promoters. There are certain constrictions in designing such vectors, and these are described here. Additionally, we review strategies for inducible shRNA expression and the various viral vectors that can be used to transduce shRNA genes into a variety of cells and tissues. The overall goal of this mini-review is to provide an overview of available approaches for optimizing RNAi mediated down regulation of gene expression in mammalian cells via RNA interference. Although the primary focus is the use of RNAi mediated cleavage of targeted transcripts, it is highly probable that some of the approaches described herein will be applicable to RNAi mediated inhibition of translation and transcriptional gene silencing.

Keywords: Dicer; siRNA; Pol III; Pol II; Inducible shRNA expression; Viral vector; PTGS; TGS

1. Introduction

RNA interference (RNAi) is a process, first described in the worm *Caenorhabditis elegans*, whereby the presence or introduction of long double-stranded RNA (dsRNA) in cells results in the degradation of homologous mRNA [1,2]. Long dsRNA is processed to 21–23 bp short interfering RNA (siRNA) with 2 nt 3′ overhangs by the RNAseIII-like protein Dicer [3]. These cleavage products are subsequently incorporated into the RNA-induced silencing complex (RISC) [4]. Delivery of chemically synthesized short interfering RNAs, mimicking Dicer cleavage substrates, results in sequence-specific, robust silencing of the expression of the corresponding endogenous gene [5], thus bypassing the non-specific inhibitory mechanisms elicited by longer dsRNA in mammalian cells [6]. RNAi can also be induced by endogenous expression of short hairpin RNAs (shRNAs) [7]. shRNAs are structurally related to a highly conserved class of small RNAs known as microRNAs (miRNAs) that mediate RNAi through a translational inihibition mechanism involving imperfect complementarity to sites in the 3′ UTR of target genes [8]. miRNAs are transcribed as precursors that are first processed in the nucleus by the RNaseIII protein Drosha in the Microprocessor complex [9–11]. The product of Drosha-mediated processing, pre-miRNA, is exported to the cytoplasm by Exportin 5 [12], for further processing by Dicer to the mature miRNA [13]. One of the strands is incorporated into a RISC-like silencing complex [14].

RNAi has recently become the method of choice for mammalian cell genetic analysis and has the potential to serve as a therapeutic treatment for a variety of acquired and hereditary diseases [15]. In this review, we will describe the various methodologies for eliciting RNAi by either synthetic or expressed RNAi effector molecules.

2. Synthetic siRNA-mediated RNAi

2.1. Enzymatically generated siRNA

The most cost effective and quickest method for siRNA synthesis is T7 phage RNA polymerase mediated in vitro transcription from short double-stranded oligo cassettes containing the promoter sequence immediately upstream of the siRNA strand template sequence to be transcribed [16,17]. The siRNA strands are synthesized in separate reactions and hybridized before purification. Once the template oligos are available, template preparation (annealing), in vitro transcription, siRNA annealing, and purification can be completed within 24 h. The siRNAs synthesized by this method frequently contain a GGG leader sequence (deriving from the promoter) as well as a 5′ triphosphate group [18]. The hybridized siRNA thus needs to be processed by T1 ribonuclease to remove the single stranded 5′ GGG overhang. If the siRNAs are transcribed with UU 3′ overhangs, T1 processing may be incomplete due to the potential formation of two G:U wobble base pairs with the G's of the 5′ leader sequence. Incomplete processing will result in retention of transcripts with 5′ triphosphate groups, which triggers non-specific inhibition of gene expression through the interferon pathway [18]. Although we have shown that the interferon response and RNAi are independent pathways [18], these siRNAs should be used with great caution in applications related to viral infection or innate immune responses. This approach may therefore be more appropriate for initial screening of target sites prior to validation of any results by chemically

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0014-5793/$30.00
doi:10.1016/j.febslet.2005.08.070

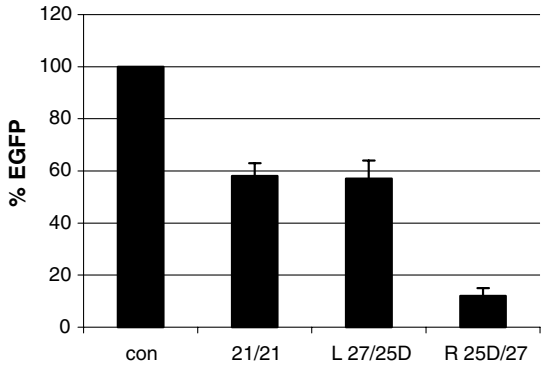
synthesized siRNAs. Replacement of the 3′ UU residues with 3′AA that cannot form wobble base pairs with the 5′GG improves processing and reduces the potential for interferon induction [18]. Changing the siRNA from 19 + UU to 21 + AA, with a 21 nt target complementary duplex region was also associated with enhanced RNAi activity.

Different siRNA sequences display widely differing efficacies, requiring screening of multiple sequences [19–21]. One way to get around this problem is by application of a pool of enzymatically generated siRNAs. Dicer, an RNase III family enzyme, cleaves in vitro transcribed long dsRNA into a pool of siRNAs suitable for gene silencing [22]. Therefore, several groups have produced a recombinant version of Dicer and used it to digest in vitro transcribed dsRNAs into a complex pool of siRNAs (d-siRNA) [23]. Nearly every pool of d-siRNAs is capable of eliciting specific gene silencing. This approach eliminates the need to identify an individual effective siRNA and has proven to be useful for transiently silencing many endogenous genes in several types of cells. Although the method is efficacious, cost effective, and relatively quick, there are some potential problems. Any residual unprocessed long dsRNA will activate RNA-dependent protein kinase (PKR), resulting in non-specific translational inhibition [6]. Gel purification of 21–23mer siRNAs from unprocessed long dsRNAs and partially processed products is therefore essential before transfection of siRNAs into cells. Additional concerns associated with the use of pools of siRNAs are the potential increased off-target effects occasionally observed with siRNA [24]. Competition from less efficacious siRNAs in a pool may also reduce the overall efficacy compared to utilization of one optimal siRNA sequence. A further advantage of utilizing one siRNA of known sequence is that any observed phenotypes of the siRNA can be verified through the application of a second siRNA species targeting the same gene. The siRNA pool approach may require additional confirmation through the use of target-specific and mismatched siRNA of defined sequence to verify the sequence specificity of the observed phenotype.

2.2. Chemically synthesized siRNAs

Chemically synthesized siRNAs represent the gold standard for RNAi applications. They are of a uniform composition and can be synthesized at higher amounts and with a wider range of chemical modifications than by other methods [25–27]. The disadvantages include higher cost and increased synthesis time. Initial studies in *Drosophila melanogaster* embryo lysates concluded that 21 nt siRNAs with 2 nt 3′ overhangs were the most efficient triggers of sequence-specific mRNA degradation [28], and most subsequent studies have therefore employed this format. During investigation of cellular interferon induction caused by in vitro transcribed siRNAs, we observed that some siRNAs of length 25–27 appeared to have greater potency than synthetic 21mer siRNAs directed to the same target site [18]. Synthetic RNA duplexes of varying length containing 3′-overhangs, 5′-overhangs, or blunt ends, were tested for their relative potency in several reporter systems [29]. Using duplex RNA at several concentrations, we observed that potency increased with length up to a duplex length of 27 bp. Increased potency was observed even for siRNAs with 5′ overhangs or blunt ends [29]. Reduced efficacy was observed for siRNA with longer than 27 bp stems, which also exhibited slower in vitro Dicing kinetics. Importantly, the 27mers do not induce interferon or activate PKR. Hannon and colleagues [30] also found synthetic shRNAs with 29-base-pair stems and 2-nucleotide 3′ overhangs to be more potent inducers of RNAi than shorter hairpins. Maximal inhibition of target genes was achieved at lower concentrations and silencing persisted longer. The improved efficacies of the longer forms of siRNA, termed "disRNAs" or "Dicer-substrate siRNAs", is postulated to result from their recognition and cleavage by Dicer, followed by their subsequently more efficient incorporation into the RISC complex. This interpretation is supported by observations that Drosophila Dicer is not only instrumental in handing over siRNA to nascent RISC, but is itself a component of the latter [31,32]. Providing the RNAi machinery with a Dicer substrate therefore presumably results in more efficient incorporation of the active 21mer into RISC. DisRNAs have subsequently been successfully employed by others [33].

Further investigation determined that the efficacy of dsiRNAs varies with the target (Kim et al., unpublished data). One reason for this is that multiple 21mer siRNAs of potentially highly variable activity can result from the same 27mer after processing by Dicer. We investigated this possibility by electrospray ionization mass spectrometry (ESI MS) analysis of in vitro diced dsiRNA. As expected, multiple 21–22mer products were generated from the dicing reactions. Optimal design of Dicer substrate siRNAs thus requires the ability to either predict the resulting 21mer(s) or direct cleavage to generate only a desired 21mer. The natural role of Dicer in cells appears to be finalizing the processing of miRNA [13], a highly conserved class of small RNAs that function in the regulation of expression of a wide range of genes at the translational level [34]. The substrates of Dicer, pre-miRNA, are bulged stem-loop structures with 2 nt 3′ overhangs, and recent reports suggest that the overhangs in the open end of the stem in such structures are bound by Dicer and determine the direction of processing [30]. The miRNA strand harboring the 3′ overhang is utilized more frequently than the top strand [35]. In an attempt to introduce a similar directionality into disRNA, we developed a format of 27mer (25/27R) in which the top strand is 25mer, and the bottom strand a 27mer with 2 nt overhangs in 3′ end. This reduced the complexity of dicing products but did not result in a single product. Further investigations determined that incorporation of DNA nucleotides in the 3′ end of the top strand (near the blunt end of the duplex) resulted in processing proceeding exclusively from the overhang end, producing a single primary 21mer siRNA of predictable sequence (Fig. 1). It is therefore possible to design a disRNA that is processed by Dicer to yield a specific, desired 21mer species. The same 21mer could be generated from a disRNA of slightly different sequence in which the top strand is 27mer with 2 nt overhangs in the 3′ end, while the bottom strand is a 25mer (27/25L), and the DNA nucleotides were introduced in the bottom strand near the blunt end. The 25/27R is processed to the 3′ end (right direction) and produces one major 21mer (R form). The 27/25mer is processed in the left direction and produces the same 21mer (L form). Interestingly, the "R" versions of the asymmetric 27mers were consistently more efficacious than the "L" versions (Fig. 1). This difference in potency of disRNA producing the same 21mer duplex suggested to us the possibility that Dicer processing may introduce some asymmetry in strand incorporation into RISC through preferential binding to the 3′ overhang. RISC-mediated

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5′ caagctgaccctgaagttcatctgcaccaccggcaa  EGFP Site-1


5′         ACCCUGAAGUUCAUCUGCACC          EGFPS1 21/21
3′       ACUGGGACUUCAAGUAGACGU

5′         pACCCUGAAGUUCAUCUGCACCACcg     EGFPS1 R 25D/27
3′       ACUGGGACUUCAAGUAGACGUGGUGGC

5′  AAGCUGACCCUGAAGUUCAUCUGCACC          EGFPS1 L 27/25D
3′  ttCGACUGGGACUUCAAGUAGACGUp
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Fig. 1. Suppression of EGFP fluorescence by RNA duplexes. Top: an EGFP expression plasmid was transfected into cells with an irrelevant (con) or the indicated siRNA at 500 pM and EGFP fluorescence measured 48 hours post-transfection. Bottom: EGFP target sequence (S strand) is shown and duplexes employed in transfections are aligned beneath in duplex form with S strand top (5′ → 3′) and AS strand bottom (3′ → 5′). RNA bases are upper case, DNA bases are lower case bold.

recognition and cleavage of the target mRNA is preceded by unwinding of the siRNA and loss of one of the strands [36], in a process probably mediated by an RNAi-associated helicase [37]. Results from Zamore's group indicate that the sequence and structure of siRNA determines which of two strands enters RISC (the guide strand) and which is excluded (the passenger strand) [38]. By taking advantage of this functional asymmetry, one can design siRNA with improved efficacy and reduced passenger strand-mediated off-target effects. We proposed that a similar mechanism could explain the differential potencies of R and L form disRNAs. Thus, even though the same 21mers are produced, 25/27R would favor incorporation of the bottom (guide) strand, while 27/25L would favor top (passenger) strand incorporation. One prediction of this postulate is that the L-form disRNA would target an antisense transcript more efficiently than the R form. We decided to test this by generating two reporter constructs in which the same cDNA fragments were cloned in two different orientations in the same position within the 3′ UTR of the Renilla luciferase gene, in the psiCheck2 vector (Promega) (Fig. 2). Cotransfection experiments determined that, for two different target genes, the L form did indeed target the antisense transcript more efficiently than the R form (Fig. 2). In conclusion, the new asymmetrical disRNA format results in 21mers of predictable sequence, superior potency, and reduced potential for passenger strand mediated off-target effects, through Dicer-dependent preferential incorporation of the guide strand into RISC.

3. Vector-based RNAi

Downregulation of gene expression mediated by siRNA is transient, and frequently lasts for only 3–5 days in cell culture [20]. While this may be sufficient for many applications, for studies of proteins with long half-lives, a single transfection of siRNA may not provide a sufficient window of functional depletion. Another potential problem inherent in transient transfection of siRNA for functional genomics studies is variability in transfection efficiency. This is of particular concern when working with difficult-to-transfect cell lines. The solution to this problem is stable expression of RNAi effector molecules from plasmids or viral vectors. The use of viral vectors, such as lentiviruses and adenovirus [39–43], allows easy generation of transgenics of even hard-to-transfect cells. Vector-based RNAi also permits co-expression of reporter genes such as GFP or luciferase, which facilitates tracking and/or selection/enrichment of transfected/transduced cells. Three different strategies exist for vector-based RNAi, involving the expression of molecules that can be classified as shRNA, siRNA and miRNA (Table 1). The most commonly used approach involves RNA polymerase III-mediated transcription of short hairpin structures with a stem of 19–29 bp and a short loop of 4–10 nt [7,44,45] (Fig. 3A). Less commonly, the two strands of an siRNA are transcribed from separate expression units, from either the same or two separate plasmids [46,47] (Fig. 3B). Finally, the effector molecules may be expressed as a chimera of siRNA and miRNA [48] (Fig. 3C).

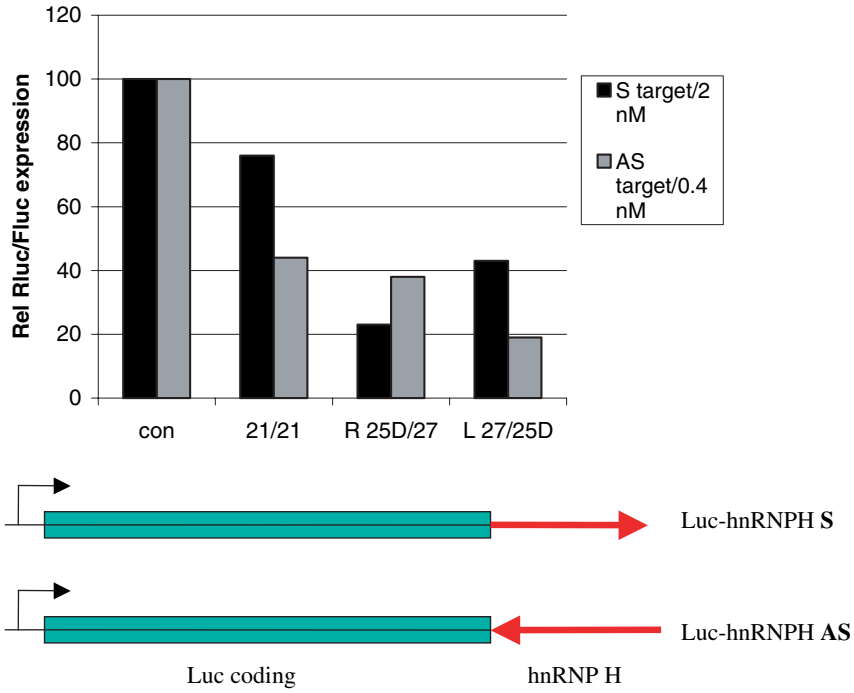

Fig. 2. Strand bias is introduced by the direction of Dicer processing. Top: Luciferase reporter constructs containing a fragment of hnRNPH coding sequence cloned into the 3′-UTR in both "S" and "AS" orientations were transfected into cells with irrelevant (con) or indicated target-specific siRNA at 0.4 or 2 nM concentration and dual luciferase activity determined 26 hours post-transfection. Relative expression levels were normalized to levels in control-transfected cells. Bottom: Luciferase reporter constructs containing a fragment of hnRNPH coding sequence cloned into the 3′-UTR of Renilla luciferase of the dual luciferase psiCheck2 vector in both "S" and "AS" orientations.

Table 1
Expression systems for vector-based RNAi

Expression unit	Promoter	Inducible system	Refs.
Chimeric miRNA	CMV	Not inducible	[48,52,53]
tRNA-shRNA fusion	tRNA-Val	Not inducible	[54]
shRNA	U6	Not inducible	[45,56]
		Tetracycline	[57,68,69]
		Ecdysone	[73]
		Cre-loxP	[74–77,79,80]
	H1	Not inducible	[7]
		Tetracycline	[60]
		Lac	[70]
	7SK	Not inducible	[57]
		Tetracycline	[57]
siRNA	U6	Not inducible	[46,47]

3.1. Expression strategies for vector-based RNAi

Expression of an shRNA as part of a longer polymerase II derived transcript faces the problem that extraneous sequences might render the heterogeneous transcripts unrecognizable by the cellular RNAi machinery [45]. This is not surprising since near-perfect stem-loop structures are not uncommon in naturally occurring mRNAs, yet do not appear to be processed to siRNA-like molecules. The expression of RNAi effectors as part of more complex transcripts therefore needs to address the problem of proper processing, which may be achieved through incorporation in the primary transcript of naturally occurring signal sequences to direct their processing. Such a strategy is used for expression of siRNA as a part of a polymerase II miRNA transcript. miRNAs are structurally very similar to siRNAs and are incorporated into a RISC-like complex that shares many of the same components as RISC [14]. Silencing by miRNA occurs at the translational level through imperfect mismatches with the target [49,50]. When the target is perfectly complementary, however, miRNAs can mediate cleavage [51]. Mature miRNAs can be generated from RNA polymerase II transcribed mRNAs containing irrelevant sequences in addition to the predicted pre-miRNA precursor sequence [48,52]. While production of mature miRNA requires maintaining the proper precursor stem-loop structure, the exact sequence of this precursor does not appear to be important, and can therefore be replaced with a heterologous stem (Fig. 3C), enabling the generation of a miRNA-based expression cassette with generalizable targeting properties. Recent work suggests that single stranded extensions to the pre-miRNA hairpin structure are required for full Drosha functionality [53]. Therefore, to ensure that heterologous miRNAs are properly processed, miRNA-based expression cassettes should, in addition to the pre-miRNA structure, contain 5′ and 3′ extensions derived from the wild-type miRNA gene to mimic the structure of the wildtype transcript as closely as possible.

Another enticing possibility is the expression of shRNA as a 3′ fusion with a tRNA to allow efficient cytoplasmic delivery while supporting the eventual removal of the tRNA component. A fusion construct between $tRNA^{Val}$ and an shRNA with a 30 bp stem has been reported to support RNAi [54], but is it unclear whether the chimeric transcript is transported

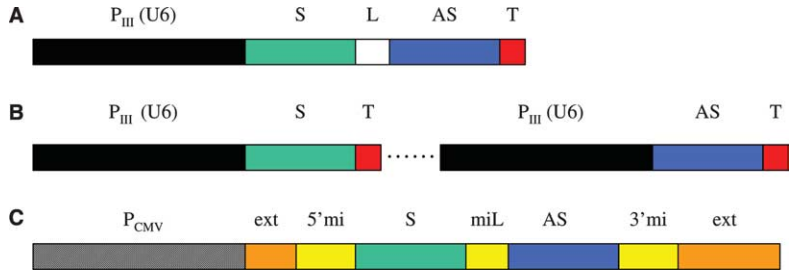

Fig. 3. Schematic representation of expression cassettes for shRNA (A), siRNA (B) and miRNA (C). P_{III}(U6): pol III promoter (U6), P_{CMV}: pol II promoter (CMV), S: siRNA sense strand, AS: siRNA antisense strand, L: loop, T: terminator, 5′mi: 5′ pri-miRNA sequence, 3′mi: 3′ pri-miRNA, ext: extraneous transcript sequences. The 5′mi and 3′mi sequences direct proper excision of the siRNA from the heterologous transcript.

intact into the cytoplasm, or is processed in the nucleus. The general utility of this approach for expression and cytoplasmic delivery of siRNA, and its potential advantages over other, simpler modes of expression, are however still open questions. Nevertheless, the recent report that a predicted virus-encoded miRNA appears to be expressed as a tRNA fusion transcript [55], validates the potential utility of this approach.

Due to the various limitations associated with the expression of RNAi effectors as parts of larger transcripts, the most common strategies for vector-based RNAi involve the use of self-contained RNA polymerase III promoters. shRNA and siRNA are commonly expressed from U6 [45,47,56], H1 [7] or 7SK [57] promoters. Transcription is initiated at a precise position outside of the promoter sequence and terminates upon encountering a stretch of 4–6 thymidines in the expression cassette. Thus, for expression of an shRNA, an expression cassette encoding, in the following order, the top strand of the hairpin, the hairpin loop, the bottom strand of the hairpin, and the terminator, is inserted immediately downstream of the promoter, by various means (Fig. 3A). For expression of siRNA, two separate cassettes consisting of the promoter, the top or bottom strand and the terminator have to be generated (Fig. 3B). Previous experiences from expression of ribozymes suggest that heterogeneity in the 3′ overhang of the transcribed shRNA may be generated, either through imprecise termination or the action of 3′ exonucleases following termination after the fourth U in the terminator [58]. This variability is however not likely to be of significant practical importance for the efficacy of the siRNAs or shRNAs, as short 3′ overhangs of variable length appear to be well tolerated within both types of molecules [28,59].

3.2. Practical considerations for construction of polymerase III-based RNAi vectors

Two principle methods for generating siRNA/shRNAs expression cassettes exist, each with its own advantages and drawbacks. In the first method, the cassette is generated by annealing of two complementary oligos, generating a double-stranded oligo cassette with appropriate overhangs for directional cloning downstream of the promoter. This methodology is straightforward and efficient, but the overhang sequences that are used for cloning will result in expression of an shRNA with a 5′ leader sequence if the restriction site in the vector that is used for cloning is outside of the promoter. Such a leader sequence is likely to affect the potency of the transcribed siRNA. This problem can be avoided by inserting the cloning site within the 3′ end of the promoter, covering positions −5 to +1 relative to the transcription start site (+1), so that the overhang from the oligo cassette will be part of the promoter proper (covering positions −4 to −1), while transcription starts at the first position in the double-stranded part of the oligo cassette, encoding the effector molecule. This strategy requires mutating the wild-type promoters to introduce a suitable cloning site. Targeted mutations within U6 and H1 promoters to introduce a *Bgl*II site have been shown to be compatible with effective transcription and shRNA-mediated silencing of expression [60] (Amarzguioui et al., unpublished data). An alternative but more cumbersome methodology, that avoids a leader sequence while retaining the wild-type promoter sequence, requires cloning of an oligo cassette with a blunt 5′ end into a recessed restriction site immediately after the promoter [47]. In addition to the oligo cassette-based cloning strategies, a PCR-based cloning strategy is also commonly employed [61]. A PCR product containing the promoter and the sequences encoding the shRNA or siRNA is amplified using a 5′ promoter primer in combination with a tagged 3′ promoter primer in which the tag consists of the reverse complement of the expression unit. This method has the advantage that the resulting PCR products support expression of siRNA or shRNA when transfected directly into cells, and the approach is therefore useful for screening of multiple constructs for efficacy [61]. However, in our experience, this approach is generally associated with a higher degree of deletion mutants, requiring more extensive screening of resulting clones.

3.3. RNAi effector molecule design

Due to the sequence-dependent variability of siRNA efficacy [20,62], design of the effector molecules is an important factor to consider. Statistical analyses of increasingly larger groups of sequences have however resulted in the identification of design rules that substantially improve the frequency of functional siRNA [38,62–64]. The single most important determinant of siRNA efficacy appears to be an asymmetry in duplex end stability that mirrors that observed for naturally occurring miRNAs and which influences asymmetrical strand incorporation into RISC [38]. Additional position-specific determinants of unknown function [63,64], as well as target secondary structure [65], also appear to contribute to overall siRNA efficacy. Although a large-scale statistical analysis of factors affecting shRNA efficacy have not yet been published, limited comparisons of siRNA and shRNA targeting the same sites suggest a similar degree of efficacy and sequence-dependence. The available evidence thus suggests that shRNA design may be based on the design rules for siRNA. In the case of U6-based expression platforms, the presence of a G at the transcription start position is highly recommended. This does, however, not rep-

resent a serious limitation, since a G in the first position of the sense strand of siRNA (the first transcribed nucleotide) is positively correlated with siRNA functionality [64].

Most design rules for siRNA have been based on duplexes of 19 bp with 2 nt 3′ overhangs, while the stems of expressed shRNAs range in size from 19 to 29 bp [7,44,45]. Early reports suggested that longer stems were generally more favorable than shorter ones [45]. A recent report analyzing the in vitro Dicer processing pattern of shRNA of various stem lengths largely confirm previous conclusions [30]. The results from these studies indicate that cleavage of the duplex occurs in a precise manner 21–22 nt from the open end of the stem. Hairpins of different lengths with extension of the duplex towards the loop would thus be expected to generate the same processed product, and any differences in efficacy between the precursor hairpins should reflect differences in either Dicer-mediated processing or cytoplasmic transport of the precursor. While differential transport efficiency cannot be discounted, the recent observations that asymmetrical siRNA of extended stems display similar improvements in efficacy as shRNA [29,66], suggest that enhanced Dicer-mediated processing contributes chiefly to this improvement. Furthermore, Dicer processing appears to also confer an asymmetry in strand incorporation into RISC, as the strand bearing the 3′ overhang (the bottom strand in an expressed hairpin) is utilized preferentially [66]. The above combined data thus suggest that shRNA design should proceed according to the following steps:

1. Select the desired 21mer siRNA sequence using the most current design rules.
2. Extend the above sequence towards the 3′ end of the target, for a duplex length of 25–29 bp.
3. Cap off the 3′ end of the duplex (the bottom strand being the guide strand) with a loop, preferably one derived from a naturally occurring miRNA.

3.4. Inducible expression of RNAi effectors

Constitutive knockdown of gene expression is not possible when the target gene is essential. Clonal differences and other counter-selection events occurring during the selection process may also complicate the interpretation of results even in cases when loss of target gene expression is not lethal. The above limitations can be circumvented through inducible expression of the RNAi effector molecules. The last two years has seen a rapid development of various methodologies for inducible expression of shRNAs from polymerase III promoters. The first method to be described was based on the tetracycline-inducible system [67]. A tetracycline-inducible H1 promoter was generated by replacement of a 19 bp sequence between the TATA box and the transcription start site with a binding site (tetO) for the tetracycline repressor [60]. In transgenic cells expressing the repressor, repressor binding to the tetO site blocks transcription, while addition of the inducer tetracycline or its derivative, doxycycline, results in dissociation of the repressor, allowing transcription to proceed. A similar strategy has been employed to generate Tet-responsive U6 [57,68,69] and 7SK [57] promoter based silencing vectors. Furthermore, replacement of a 26-nt sequence between the TATA box and the transcription start site in the H1 promoter with a lac operator, results in IPTG-responsive shRNA expression in transgenic cells expressing the lac repressor [70]. A major advantage of the above systems is reversibility of knockdown. Thus, reemergence of target gene expression has been demonstrated to occur within 3–4 days of withdrawing the inducer for both doxycycline-inducible U6 [68] and lac-responsive [70] H1 expression systems. These doxycycline-inducible expression systems are readily applicable in vivo, as administration of non-toxic concentrations of doxycycline in the drinking water of experimental animals results in silencing of in vivo target gene expression [71].

The tet-responsive polymerase III promoters display some level of leakiness, which may result in significant downregulation even in the absence of induction when working with very potent shRNA [69] (Amarzguioui et al., unpublished data). A more tightly regulated U6 promoter was recently described. This expression system contains two optimally placed tet operators and displays a combination of low basal transcriptional activity and effective silencing in the induced state [69]. An alternative to the tetracycline-inducible system is the more tightly regulated but generally less active ecdysone-inducible system [72]. Ecdysone (muristerone A)-inducible expression of shRNA under a modified U6 promoter, in which the U6 enhancer was replaced with a GAL4 element, has been shown to facilitate efficient inducible silencing of target gene expression in cells expressing a GAL4-Oct-2^{Q} transactivator fusion and the nuclear receptor/transcription factors VgEcR and RXR [73]. Addition of the ecdysone analogue initiates an activation cascade involving dimerization of the transgenic transcription factors, activation of GAL4-Oct-2^{Q} transactivator expression, and finally, activation of the modified U6 promoter. shRNA-mediated downregulation of gene expression was reversible, as target gene expression recovered gradually from 2 to 4 days after withdrawal of the inducer [73].

Recently, Cre-LoxP recombination based systems for conditional shRNA-mediated downregulation of gene expression have been reported [74–77]. This approach involves the insertion of a loxP-flanked stuffer sequence between the DSE and PSE [76] or PSE and TATA box [75] elements within the U6 promoter to disrupt the proper spacing between these elements, thereby inactivating the promoter. Expression of the Cre protein, either from an inducible endogenous locus [76] or by exogenous lentiviral-mediated delivery [75], results in recombination between the two loxP sites and removal of the intervening stuffer sequence, which brings the different elements of the promoter together, thereby generating an active promoter. The disadvantage of the Cre-loxP system for condition expression compared to the other inducible systems is that it involves genomic rearrangements, and is therefore irreversible. This system has substantial potential for in vivo spatiotemporal control of gene expression, due to the existence of a wide range of transgenic mouse strains expressing Cre in different tissue and at different stages of development [78]. The effectiveness of this system in vivo has already been demonstrated [77,79,80]. Especially encouraging is the demonstration for the first time of combined Cre recombinase and tetracycline-dependent tissue-specific knockdown of gene expression in transgenic mice carrying a Cre recombinase gene under the control of a liver-specific tetracycline-responsive human albumin promoter [79]. Liver-specific expression of Cre, resulting in activation of shRNA expression, could therefore be induced simply by administration of the tetracycline-analogue doxycycline in their feed. This approach in combination with the many existing strains of Cre transgenic mice, will allow the rapid development of conditional shRNA-expression based mouse models for human genetic diseases.

4. Conclusions and summary

RNAi has emerged as one of the most interesting and important mechanism for sequence-specific downregulation of gene expression at both the transcriptional and post-transcriptional levels. In essence, RNAi has enabled a number of heretofore impossible genetic analyses to be conducted in mammalian cell culture, and now in transgenic animals. The explosion in the applications of RNAi for targeted gene knockdown has fueled ingenious and very useful methodologies for design and delivery of small interfering RNAs and their precursors, as well as siRNA or shRNA gene expression units. Aside from widespread applications in gene analyses and target validation in mammalian cells and experimental animals, RNAi is emerging as a potential therapeutic modality as well. Of utmost importance is the fact that a major percentage of the human transcriptome is proposed to be regulated post-transcriptionally by miRNAs. A better understanding of this important epigenetic process will certainly translate into more efficacious siRNA experiments and applications. The recent discovery of transcriptional gene silencing mediated by small RNAs also will open many new possibilities for modulating gene expression in mammalian systems. Those of us studying the mechanisms and applications of RNAi all have the feeling that there are many more exciting discoveries and applications of small RNAs yet to come.

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